INHIBITION OF NADP-LINKED MALIC ENZYME BY GLYOXYLATE

Juan José CAZZULO and Esther MASSARINI
Departamento de Bioquímica, Facultad de Ciencias Bioquímicas, Universidad Nacional de Rosario, Suipacha 570, Rosario, República Argentina

Received 20 January 1972
Revised version received 15 February 1972

1. Introduction

The function of NADP-linked malic enzyme (L-malate: NADP oxidoreductase (decarboxylating) EC 1.1.1.40) is supposed to be both degradative, when microorganisms grow on C_4-dicarboxylic acids, and biosynthetic, yielding NADPH and pyruvate [1].

Acetyl-CoA, which derives from pyruvate, is an inhibitor of the malic enzyme from *Escherichia coli* [2]. The enzyme in *Pseudomonas putida* is not inhibited by acetyl-CoA but is repressed during growth on acetate, ensuring the conservation of C_4-dicarboxylic acids required for the glyoxylate and tricarboxylic acid cycles [3].

The NADP-lined malic enzyme from a marine Pseudomonas was not repressed by acetate, and was rather poorly inhibited by acetyl-CoA; it was strongly inhibited, however, by low concentrations of glyoxylate. If we consider glyoxylate as a product arising from acetyl-CoA through the action of citrate synthase, aconitase and isocitrate lyase, the inhibition of malic enzyme by glyoxylate could have a similar physiological significance.

2. Materials and methods

Glyoxylate, L-malate, deoxyribonuclease I and DEAE-cellulose were purchased from Sigma Chemical Co., St. Louis, Mo.; NADP and CoA from Boehringer, Mannheim. Acetyl-CoA was synthesized from CoA and acetic anhydride by the method of Stadtman [4] and assyed with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) [5].

The marine Pseudomonas was grown at 20° in 1.5 l batches of complex medium [7] prepared with undiluted artificial sea water [8], shaken and aerated by air bubbling. When the absorbance of the culture at 680 nm reached about 1.4 the cells were harvested by centrifugation at 25,000 *g* for 30 min at 4°, washed with 0.05 M Tris-Cl buffer pH 7.6, containing 0.5 M NaCl and 1 mM EDTA, and suspended in 0.05 M Tris-Cl buffer pH 7.6, containing 0.2 M KCl and 1 mM EDTA (2.7 ml per g of cells, wet wt). The cells were disrupted at 5° by 2 treatments (15 sec each) in a MSE sonic disintegrator (Measuring and Scientific Equipment, Ltd., London, England) at maximum power. To the homogenate was added $MgCl_2$ (2 mM final conc.) and deoxyribonuclease I (7 μg per ml of suspension) and it was centrifuged at 25,000 *g* for 40 min at 4°. The supernatant fluid was fractionated by treatment with solid ammonium sulphate; the 50–70% saturation fraction, containing most of the malic enzyme activity (specific activity 0.308 μmoles of NADPH formed/min/mg of protein) was dialysed for 4 hr against 20 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, and applied to the top of a column of DEAE-cellulose (1.5 × 20 cm) equilibrated with the same buffer. A linear gradient of KCl was applied, from 0 to 500 mM, the malic enzyme being eluted between 260 and 300 mM K^+, as determined by flame photometry. The fractions containing the higher specific activities were pooled and dialysed for 4 hr against 0.05 M Tris-Cl buffer pH 7.6, containing 0.1 mM EDTA. The specific activity of the enzyme in the dialysed pool, which was used for all the experiments reported here, was 20.32 μmoles of NADPH

North-Holland Publishing Company – Amsterdam

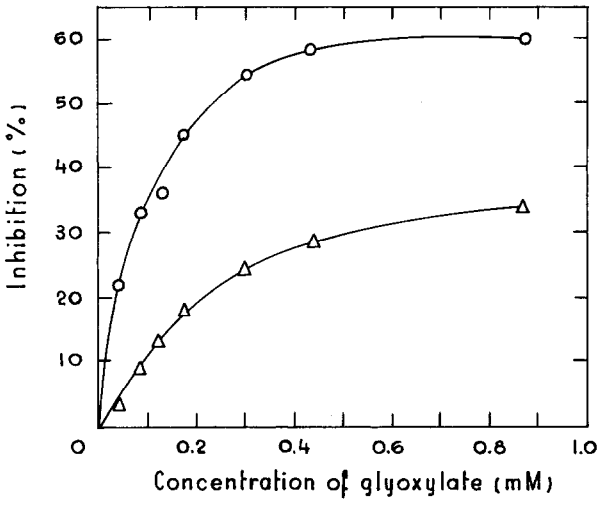

Fig. 1. Inhibition of malic enzyme by glyoxylate, at 2 concentrations of L-malate. The reaction mixtures contained (in μmoles) in a final volume of 1 ml: Tris-Cl buffer, pH 8.6, 200; $MgCl_2$, 2; NADP, 0.1; NH_4Cl, 20; glyoxylate, as stated on the abscissa. (o–o–o), 2.5 μg of enzyme and 0.1 mM L-malate-Tris; the 100% of activity was 26 nmoles of NADPH/min. (△–△–△), 1.25 μg of enzyme and 2 mM L-malate-Tris; the 100% of activity was 27 nmoles of NADPH/min. The reaction was started by the addition of the enzyme after equilibration of the otherwise complete reaction mixture in the chamber of a Beckman DB-G recording spectrophotometer at 22° for 1 min, and followed as the increase of absorbance at 340 nm.

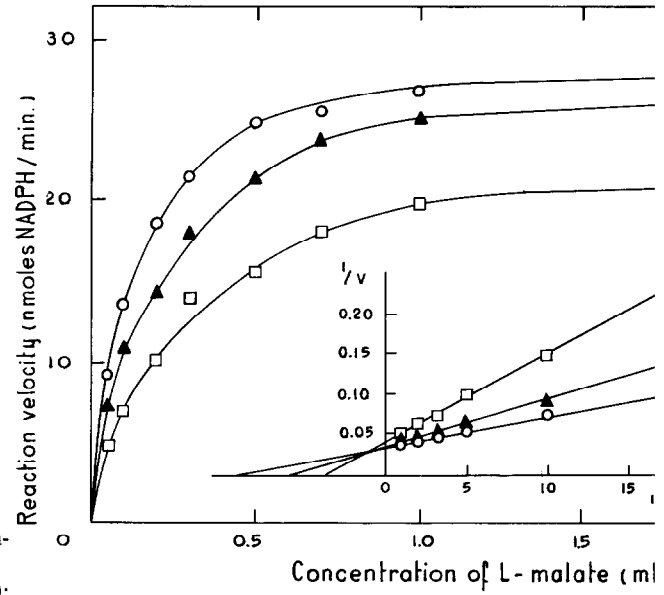

Fig. 2. Effect of glyoxylate on the saturation curve for L-malate. Experimental conditions as stated in the legend to fig. 1, except for the enzyme concentration (1.25 μg), L-malate, as stated on the abscissa, and glyoxylate, 0: (o–o–o), 0.09: (▲–▲–▲), or 0.45: (□–□–□) mM.

formed/min/mg of protein. This means a 147-fold purification with respect to the crude extract (0.138 μmoles of NADPH formed/min/mg of protein). The preparation was free of NADP-linked isocitrate dehydrogenase and lactic dehydrogenase, and contained a very low malic dehydrogenase activity.

The malic enzyme was assayed spectrophotometrically as described in the legends to figures; during the purification procedure the assays were performed at pH 7.6. Protein was determined by the method of Lowry et al. [8]. Glyoxylate was determined as described by McFadden [9] for the assay of isocitrate lyase.

3. Results and discussion

As shown in fig. 1, glyoxylate was a powerful inhibitor of the malic enzyme from the marine Pseudomonas. When the effect was tested in the presence of 0.1 mM L-malate (concentration near the apparent K_m for this substrate) 50% inhibition was attained at about 0.25 mM glyoxylate, whereas in the presence of 2 mM L-malate (approximately 18 times the apparent K_m) the maximal inhibition attained (with 0.87 mM glyoxylate) was about 30%. The effect of 0.45 mM glyoxylate was independent of the pH of the reaction mixture between 6.9 and 9.1, and was not an apparent inhibition due to reaction of the glyoxylate itself with the NADPH formed in the reaction. NADPH was not oxidized by the enzyme preparations used in the presence of glyoxylate, and direct chemical determination of glyoxylate in reaction mixtures showed no appreciable destruction of the inhibitor. The inhibition was reversible, since it disappeared upon dilution of a sample preincubated with 1 mM glyoxylate for 10 min either at room temp or at 50°.

The product pyruvate, even at 1 mM, did not inhibit the enzyme under conditions similar to those stated in the legend to fig. 1. Sanwal and Smando [10] [10] tested 0.4 mM glyoxylate as an analogue of gly-

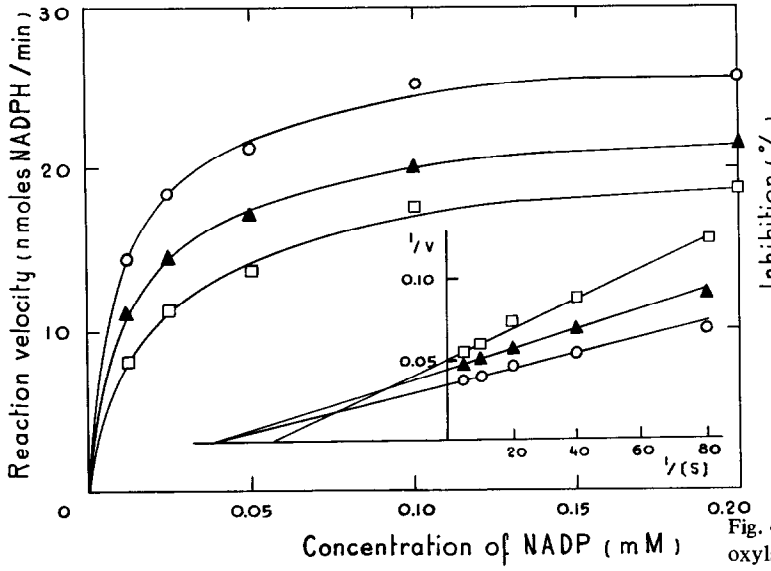

Fig. 3. Effect of glyoxylate on the saturation curve for NADP. Experimental conditions as stated in the legend to fig. 2, except for the concentration of L-malate, which was 1 mM, and NADP, which varied as stated on the abscissa. Glyoxylate, 0 (○–○–○), 0.09 (▲–▲–▲) or 0.45 (□–□–□) mM.

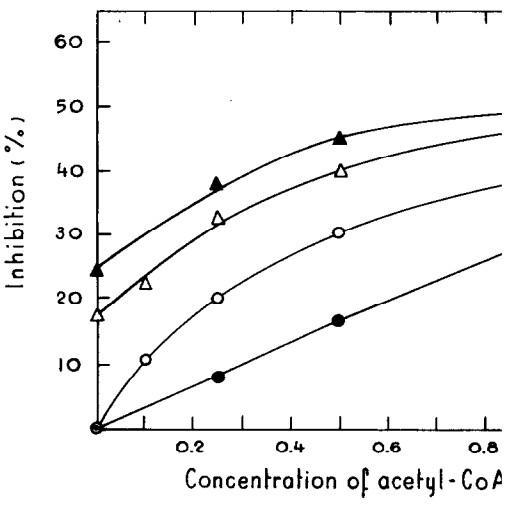

Fig. 4. Inhibition of malic enzyme by acetyl-CoA and gly oxylate. Experimental conditions as described in the lege to fig. 1, except for the concentration of L-malate (0.1 n the amount of enzyme (1.25 μg) and the pH of the react mixtures and the concentration of the inhibitors, which as stated below. (○–○–○) and (●–●–●), % of inhibition function of the concentration of acetyl-CoA, in the abse of glyoxylate, at pH 7.5 or 8.6, respectively. (△–△–△) ar (▲–▲–▲), the same, but in the presence of 0.09 mM glyo te. The 100% of activity was 11 nmoles of NADPH/min, pH 7.5, and 16 nmoles of NADPH/min, at pH 8.6.

cine for the desensitization of the malic enzyme from *E. coli*, and found an inhibition of 18%. The malic enzyme partially purified from *Enterobacter cloacae* was inhibited by glyoxylate to a similar extent (Cazzulo and Massarini, unpublished results).

The experiment of fig. 1 suggested that the inhibition by glyoxylate was directly related to the concentration of the substrate L-malate. This is further demonstrated in fig. 2. The plots of reaction velocity against concentration of L-malate were hyperbolic, either in the presence or in the absence of the inhibitor. The presence of the latter affected mainly the K_m value for L-malate, which changed from 0.113 mM (no glyoxylate) to 0.178 mM and 0.277 mM (0.09 and 0.45 mM glyoxylate, respectively). The V_{max} showed only little variation. The inhibition was not strictly competitive, since at 2 mM glyoxylate (not shown in fig. 2) the K_m value was further increased to 0.384 mM, but the V_{max} fell to about two thirds of the value in the absence of the inhibitor.

When the effect of glyoxylate on the saturation curves for NADP at 1 mM L-malate was studied (fig. 3) again the curves were hyperbolic, but in this case the V_{max} was more affected than the apparent K_m, w varied from 0.014 mM (no glyoxylate) to 0.015 a 0.018 mM (0.09 and 0.45 mM glyoxylate, respect

The inhibition of the malic enzyme from the n Pseudomonas by glyoxylate seems not to be allos in nature. The saturation curves for glyoxylate its as well as those for the substrates in the presence the inhibitor, were hyperbolic, and the inhibition not appreciably affected by pH. The inhibition of malic enzyme from *E. coli* by acetyl-CoA [2] sho sigmoid kinetics and depended on the pH of the r action medium. As shown in fig. 4, the enzyme fr the marine Pseudomonas was also inhibited by ac CoA, and this inhibition varied with the pH of the action mixture, being greater at pH 7.5 than at pH It is noteworthy that acetyl-CoA, even at the low pH value, was a considerably poorer inhibitor tha glyoxylate. It is also interesting that at both pH v the effects of low concentrations of the inhibitors nearly additive (fig. 4), suggesting that *in vivo* the

ence of low concentrations of glyoxylate could enhance the inhibition by acetyl-CoA, and *vice versa;* further speculation is prevented by our lack of knowledge of the actual concentrations of both inhibitors inside the living acetate-grown Pseudomonas cell.

As pointed out by Jacobson et al. [3], growth on acetate requires a continuous synthesis of C_4-dicarboxylic acids, through the agency of the glyoxylate cycle [11], to compensate for their withdrawal for biosynthetic purposes; under these conditions, acetate repression (or acetyl-CoA inhibition) of the NADP-linked malic enzyme would result in a decreased loss of C_4-dicarboxylic acids through decarboxylation, and allow their conservation for the maintenance of the cycles. If we consider that glyoxylate arises from acetyl-CoA through the action of citrate synthase, aconitase and isocitrate lyase, the inhibition by both metabolites could have a similar physiological meaning. Since the malic enzyme from the marine Pseudomonas was less affected by acetyl-CoA than the similar enzyme from *E. coli,* and was not repressed upon growth on acetate as sole carbon source, the inhibition by glyoxylate could be physiologically significant. The acetate-grown marine Pseudomonas contained a high activity of NADP-linked isocitrate dehydrogenase, which might ensure the provision of NADPH for biosynthetic purposes independently from the malic enzyme reaction; pyruvate might be obtained through the concerted action of phosphoenol-pyruvate carboxykinase and pyruvate kinase, or by an oxaloacetate decarboxylase, since all these enzymes are present in crude extracts obtained from acetate-grown cells.

Acknowledgements

This investigation was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas, and the Consejo de Investigaciones Científicas y Técnicas de la Provincia de Santa Fé (República Argentina). JJC is a member of the Research Carreer of the former institution. The authors are indebted to Dr. R.E. Trucco for the gift of the microorganism, and to Drs. B.C. de Bracalenti and F. Seta for allowing the use of equipment belonging to their departments.

References

[1] B.D. Sanwal, Bacteriol. Revs. 34 (1970) 20.
[2] B.D. Sanwal, J.A. Wright and R. Smando, Biochem. Biophys. Res. Commun. 31 (1968) 623.
[3] L.A. Jacobson, E.C. Bartholomaus and I.C. Gunsalus, Biochem. Biophys. Res. Commun. 24 (1966) 955.
[4] E.R. Stadtman, in: Methods in Enzymology, Vol. 3, eds. S.P. Colowick and N.O. Kaplan (Academic Press, New York and London, 1957) p. 931.
[5] J.L. Cánovas and H.L. Kornberg, Proc. Roy. Soc. Lond. B 165 (1966) 189.
[6] D.A. Corlett, Jr., J.S. Lee and R.O. Sinnhuber, Applied Microbiol. 13 (1965) 808.
[7] R.A. MacLeod, E. Onofrey and M.E. Norris, J. Bacteriol. 68 (1954) 680.
[8] O.H. Lowry, N.J. Rosebrough, A.L.Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[9] B.A. McFadden, in: Methods in Enzymology, Vol. 13, ed. J.M. Lowenstein (Academic Press, New York and London, 1969) p. 163.
[10] B.D. Sanwal and R. Smando, J. Biol. Chem. 244 (1969) 1817.
[11] H.L. Kornberg, in Essays in Biochemistry, Vol. 2, eds. P.N. Campbell and G.D. Greville (Academic Press, New York and London, 1966) p.1.